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Bacterial Spores Remain Viable After Electrospray Charging and Desolvation

Sara Pratt

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

Bacterial Spores Remain Viable After Electrospray Charging and Desolvation

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Master of Science

The electrospray survivability of *B. subtilis* spores and *E. coli* was tested in atmospheric mobility experiments. *E. coli* did not survive electrospray charging and desolvation, but *B. subtilis* did. Experimental conditions ensured that any surviving bacteria were charged, desolvated, and de-agglomerated. *B. subtilis* was also found to survive both positive and negative electrospray and subsequent introduction into vacuum conditions. Attempts were made to measure the charge distribution of viable *B. subtilis* spores using electrostatic deflection. From those experiments, it was found that either the spores do not become highly charged under the electrospray conditions used or only spores in a low positive or negative charge state survive.

Keywords: electrospray, mass spectrometry, *Bacillus subtilis*, extremophiles

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1 INTRODUCTION

1.1 Electrospray of Microorganisms

Electrospray Ionization (ESI) has revolutionized access to biological molecules and systems for mass spectrometric and ion mobility analysis. The process of electrospray consists of spraying a solution of the desired analyte in a strong electric field.[1] The solution flows through a capillary and forms a Taylor cone at the end from which charged droplets are emitted. As solvent evaporates, the droplets break apart when the force of columbic repulsion overcomes the surface tension, a point known as the Rayleigh instability limit. The Rayleigh limit is expressed as $q_{R\gamma} = 8\pi\sqrt{\varepsilon_0 \gamma R^3}$ where $q_{R\gamma}$ is the maximum charge, γ is the surface tension, and R is the radius. As solvent continues to evaporate, that process is repeated, eventually leaving individual charged particles once all the solvent is evaporated.[2] This process is illustrated in Figure 1.

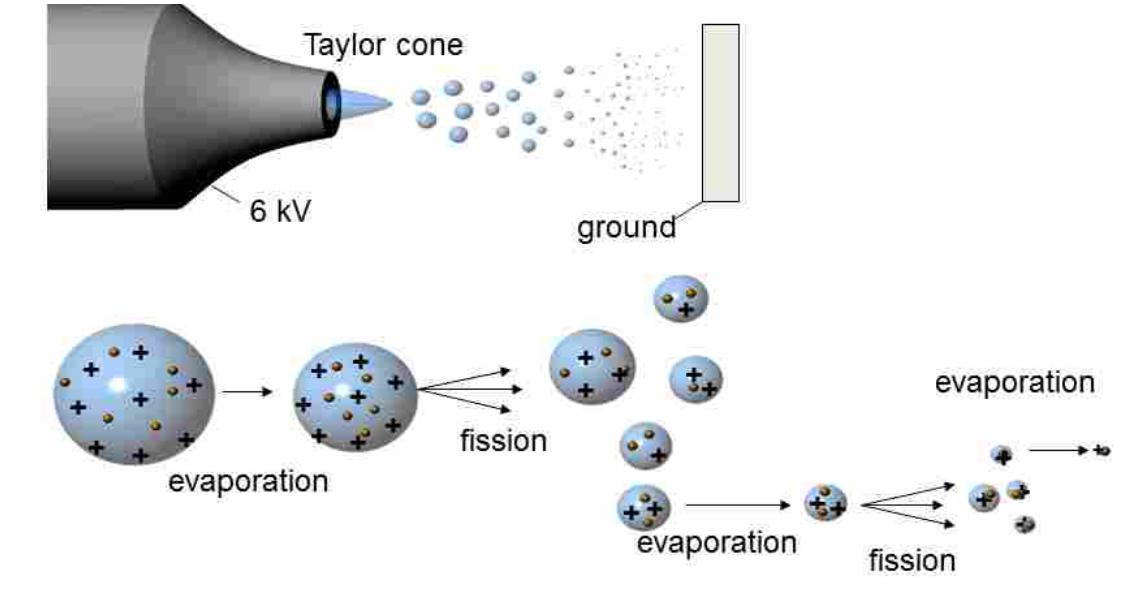

Figure 1. An illustration of the electrospray process. Courtesy of Daniel Austin.

There are two dominant theories to explain ion formation in electrospray.[2] The description of electrospray in the previous paragraph is termed the charged-residue model. The mechanism ends with a single molecule in a charged droplet that retains the charge from that droplet once the solvent evaporates. The second model is termed the ion evaporation model.[3, 4] In this model, molecules form desolvated ions by evaporating from the surface of highly charged micro-droplets. In general, the charged residue model is preferred to explain the mechanism of electrospray ionization for large molecules while the ion evaporation model is used for smaller molecules.[5] Because the bacteria are so large relative to typical analytes, the charged residue model is the preferred model to explain their ionization.

Electrospray is mainly used as an ionization technique for mass spectrometry. Mass spectrometry consists of ionizing molecules and then separating and detecting them according to their mass to charge ratio.[5] It is used to analyze all kinds of molecules including organic and inorganic compounds. Depending on the ionization technique used, the ions can be intact, fragmented, or clustered. Electrospray is a useful ionization technique because it is "soft" and keeps the analyte intact. This is especially useful for bigger molecules that get torn apart by other, harder ionization techniques. With ESI you can see the molecular ion instead of just fragments.

The study of principles governing electrospray dates back as far as the early twentieth century. Zeleny studied the effect of electric fields on the surface of liquids, mostly ethanol.[6] In his studies, he explored the voltage required to induce instability at the liquid surface. He observed the formation of a Taylor cone (although it was not then known by that name) and a thread of liquid coming out of the cone and subsequently forming droplets. Taylor studied water

drops in electric fields further in the early 1960s.[7] He described the cone formed by water in electric fields, which was subsequently named after him. In the late 1960s the Dole group explored the use of electrospray for mass spectrometry.[8] Unfortunately, they could not detect the large polystyrene ions they formed with the mass spectrometers available at the time. The Fenn group worked on electrospray for many years. In 1984 they proposed the use of electrospray to analyze large organic molecules.[9] They analyzed molecules up to 2000 u with a quadrupole mass analyzer. For his work with electrospray, Fenn was awarded the Nobel Prize in Chemistry in 2002.[10]

Although mainly used for proteins and other biomolecules, ESI has also been used to charge intact biological systems such as viruses[11-14] and bacteria.[15, 16] Some researchers have demonstrated that viruses are not only intact, but also viable after the electrospray process. Siuzdak *et al.* were the first group to test viruses for viability after electrospraying.[11] They were exploring ESI mass spectrometry as a viral analysis tool, and also wanted to see if electrospraying viruses would give insight into whether non-covalent interactions were maintained under electrospray conditions. Although they were unable to measure the masses of the viruses, they operated their quadropole in RF only mode and allowed the charged virus particles to pass to the detector where they saw a small signal. They performed rudimentary mass selection to ensure that they were collecting charged virus particles. In other experiments, electrosprayed tobacco mosaic virus particles were collected on a glycerol-coated brass plate and examined with transmission electron microscopy. The examination found that the viruses retained their quaternary structure. To further see if the native structure was retained, tobacco plants were inoculated with the tobacco mosaic virus. The viruses were viable and infected the

plants indicating that functional structure was fully retained. That research group made further progress with electrospray of viruses by using ESI-TOF with charge detection.[12] The charge detector was able to detect a much higher mass to charge ratio than their previous setup. Using that technique, they were able measure the mass of both rice yellow mottle virus and tobacco mosaic virus.

In addition to viruses, bacteria have been examined with electrospray and electrospray-based techniques. Vaidyanathan *et al.* used electrospray of whole cell suspensions of *B. subtilis* to discriminate between strains.[17] The mass spectra from electrosprayed whole cell suspensions were very similar to the mass spectra of cell-free supernatants. The lack of many additional compounds in the spectra of whole cell suspension indicates that the cells likely stayed intact. Additionally, a biofilm of *B. subtilis* was shown to be viable after desorption electrospray ionization mass spectrometry (DESI-MS).[18] The mass spectrum for that experiment was dominated by surface proteins, which implies that the cells in the biofilm were not lysed under those conditions either. If DESI-MS lysed the cells, then more interior proteins would likely be found in the mass spectrum.

Kim *et al.* used electrospray to generate viable aerosols of *Staphylococcus epidermidis* and *Escherichia coli*.[15] Although their experiments explored whether bacteria can remain viable after electrospray, these efforts did not desolvate the sprayed bacteria, exclude agglomerates, or determine whether surviving bacteria themselves were electrically charged. The aim of their work did not require answers to those questions, but they must be addressed before the applications of electrospray of bacteria can be expanded to mass spectrometry.

The main objective of the Kim group was to generate an aerosol of bacteria using electrospray.[15] They were the first group to report this. In their experiments, they electrosprayed bacterial suspensions of *Staphylococcus epidermidis*, *Escheria coli*, and a sterilized broth control. The bacteria solutions were prepared by inoculating sterilized nutrient broth liquid media. The electrospray tip was in an enclosed box filled with CO_2 to stabilize the spray with a ground plate 2 mm below it. A light source was used to illuminate the electrospray and a CCD camera with zoom lens was used to capture images and classify spray modes. The size distribution of the droplets was measured with a Doppler particle analyzer. They observed droplet sizes from 8.5 to 22.8 μm. In experiments either an Andersen impactor Z-A6 with agar plates to collect the electrosprayed bacteria or an impinge-type biosampler from SKC with sterile water collector was connected to a hole in the ground plate.

The number of charges on the bacteria was calculated from the current between the spray nozzle and ground plate using this formula:

$$N_{EC} = \frac{\pi A D_p^3}{6Qe}$$

In this equation: N_{EC} is the number of elementary charges, A is the spray current, D_p is the droplet size, Q is the flow rate, and e is the elementary charge. To measure the current, a mesh-type electrode replaced the ground plate and was connected to a nanoampere meter. As the water flow rate increased, the spray current increased. They observed charges that were approaching the Rayleigh limit, and as expected the charges increased as the droplet size increased.

To look for viability, they used the Anderson impactor. After each experiment the agar plates from the impactor were incubated for 1-2 days. They sprayed the sterile media control and

observed no growth but they did observe growth when they sprayed *E. coli* and *S. epidermis*. To quantify the proportion of the bacteria that remained intact, they used the biosampler, again incubated for 1-2 days. Around 25% of the *E. coli* survived and 3% of the *S. epidermis* survived. They attributed the loss to stress from the high electric field, loss of particles in the path, and stress of the cells from being in the gas phase.

There are a number of scenarios that would explain why the bacteria Kim *et al.* electrosprayed survived the ionization conditions other than the optimal condition of the bacteria surviving electrospray while desolvated, de-agglomerated, and charged. Although they said that the bacteria they electrosprayed were de-agglomerated, they did nothing to prevent agglomeration except relying on columbic repulsion. Even if the bacteria were de-agglomerated, they were definitely not desolvated. *E. coli* cell size is around 3 μm, which is much smaller than the droplet size Kim *et al.* observed.[19] If bacteria only survive electrospray while agglomerated, then it would not be useful for coupling culturing techniques with mass spectrometry because bacteria could not be separated based on individual mass. This is also the case if bacteria only survive while solvated. It is possible that some species could survive electrospray only when solvated because the bacteria cell itself is not experiencing an electric field. This is because all of the surface of the water droplet would be a Gaussian surface with all of the charge concentrated on the surface. This happens because the charges come to equilibrium and spread out from each other as much as possible. Also, in an aggregate of bacteria, a similar phenomenon would occur where surface bacteria would be charged and core bacteria may not be charged. An illustration of the need for desolvation and de-agglomeration is shown in Figure 2. Therefore, for the results to

be meaningful, the bacteria that survive must not be solvated or agglomerated. Further expansion of the work of Kim et al is needed.

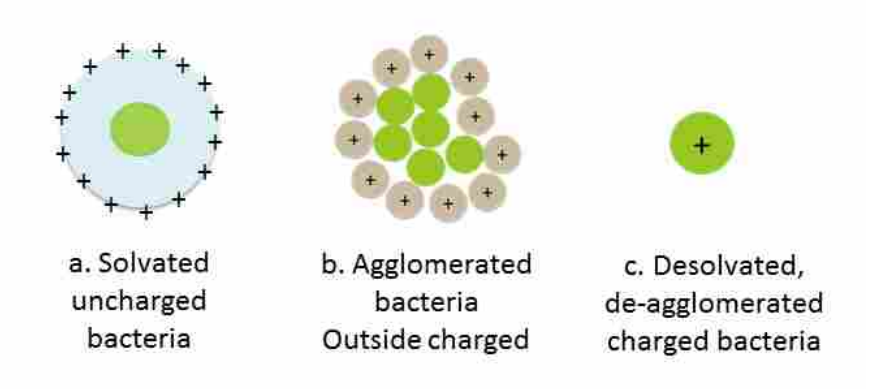

Figure 2. This figure shows three different cases in which bacteria could survive electrospray. In this figure green bacteria are alive and grey bacteria are dead. a. In this case, the bacterium survives without experiencing an electric field. b. In this case, the interior bacteria are uncharged and survive. The outside bacteria are charged and die. C. In this case, the bacterium itself is charged and survives.

The primary goal of the work in this thesis was to see whether bacteria, and which species of bacteria, can survive all the conditions of electrospray. The conditions we tested include desolvation, de-agglomeration, charging, and vacuum. Only if bacteria survive all of these conditions would electrospray of viable bacteria be useful for coupling mass spectrometry with biological techniques such as culturing.

1.2 Bacteria used in these studies

There were two species of bacteria used in these studies. One of the species used was *E. coli*, which was used by Kim *et al.*[15] The other species chosen for these studies was *Bacillus subtilis*, because it can survive many extreme conditions as an endospore. An endospore, or spore, is a tough structure formed by some bacteria, including *Bacillus subtilis*, to survive harsh environments. The process of spore formation is called sporulation. In the spore state they are

resistant to desiccation, vacuum, gamma radiation, UV radiation, oxidizing agents, dry heat, and wet heat.[20] There are many contributors to the ability of spores to resist harsh conditions. The genetic makeup of the spores, the conditions for sporulation, spore coats, core permeability, core water content, spore mineral content, α/β-Type SASP (small, acid-soluble proteins), and mechanisms for macromolecule damage repair all contribute to the spore's survival abilities.

Sporulation in *B. subtilis* is triggered by starvation, which is marked by a nutritional deficit of carbon, nitrogen or phosphorus.[21] The spore, which is metabolically inactive, has molecules and structures that the vegetative cell does not. Figure 3 is an illustration of sporulation in *B. subtilis*. Spore formation starts with a septum off to the side, dividing the cell unevenly into a smaller forespore and a bigger mother spore. The forespore becomes a protoplast with a double membrane. Then, finally, a coat is formed to protect it. After that critical structure forms around the forespore, the cell lyses and a mature spore is released.[22] The core, the inner portion which holds the DNA, is slightly desiccated, which contributes to survival.

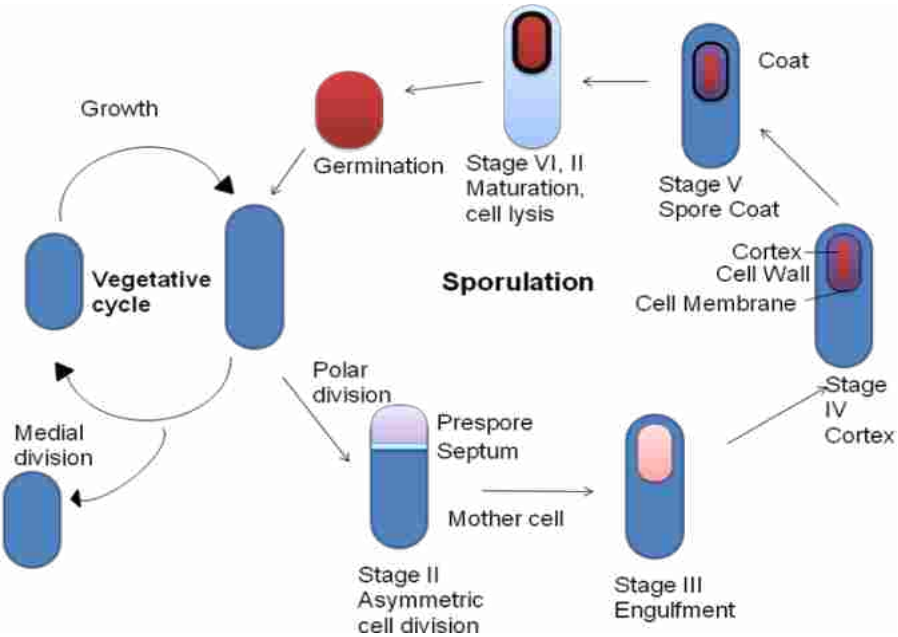

Figure 3. The sporulation process. Adapted from Regulation of endospore formation in *Bacillus subtilis* in *Nature Reviews Microbiology*.[23]

Bacillus spores are surrounded by a multilayer protein coat.[22] This coat is a significant contributor to the ability of spores to withstand harsh conditions. Chada *et al.* performed AFM experiments done in contact mode that show scan lines indicating that the spore coat may be somewhat soft. The spores are on average 1.2 µm long and 0.8 µm wide. There is a series of ridges 85± 5 nm thick and 12± 4 nm high. The ridge formation may be due to the core dehydration process.[22] The entire spore is covered by bumps that are 7 to 20 nm in diameter. There are some larger bumps, mainly on the ridge that are 20 to 40 nm in diameter. A spore is covered by about 5,500 bumps on average. The rough surface may provide extra surface area for charging. They weigh on average 196 femtograms.[24] *B. subtilis* spores may seem small, but they are actually large compared to things that are usually electrosprayed. Like all living things, they come in a distribution of sizes. Different studies have given slightly different measurements for

their dimension. Some results that differ from dimensions given previously are as follows: The length of *B. subtilis* spores is on average 1.07 μm with a typical range of 0.89–1.53 μm.[25] The diameter is on average 0.48 μm with a typical range of 0.41–0.67 μm.

1.3 Need to Test Impact Survivability

In addition to the need to further explore electrospray of bacteria from the standpoint of mass spectrometry and ion mobility spectrometry analysis, ESI charging may enable studies of bacteria survival of impacts. When looking for signs of extraterrestrial life, it is important that spacecraft don't contaminate a sample. If spacecraft crash, they could potentially contaminate other planets with bacteria and give false positive results when looking for signs of life. We aim to test whether bacteria can survive high-velocity impacts using electrospray to introduce them into an acceleration instrument.

NASA's planetary protection program is responsible for ensuring that missions to other planets do not contaminate them with biological material.[26] NASA has policies in place to reduce the likelihood that solar system missions have forward or backward contamination. Forward contamination is contamination of other planets as a result of exploring them and backward contamination is contamination of Earth as a result of bringing back samples from other planets. The impact work would be useful for studying the likelihood of forward contamination. One of the NASA mission design requirements for planetary protection is to avoid impact with the planet being studied. Previously, however, some missions have crash landed onto the surface of Mars and more may unintentionally crash on Mars or other planets in the future.[27] For missions such as rovers, only the exposed portions of the spacecraft have to

meet the stringent sterilization requirements. Upon impact, however, the other parts of the spacecraft that were not sterilized as thoroughly can come in contact with the surface of the planet. Research into which bacteria, if any, can withstand those impacts will help determine if those crashes could be of concern for biological contamination.

1.3.1 Overview of Extremophiles in Space Conditions

Many species of microorganisms can survive various extreme conditions. Many groups have tested the limits of these microorganisms, known as extremophiles, in surviving outer space conditions including vacuum and ultraviolet radiation. Cockell *et al.* reported on the outcomes of microbes that were exposed to space on the outside of the international space station for 548 days.[28] Some augmented organisms, *A. cylindrica* and *Chroococcidiopsis*, survived, as well as some natural ones. Two natural algae (*Chlorella* and *Rosenvingiella spp.*), a cyanobacterium (*Gloeocapsa sp.*) and two bacteria survived being exposed to the vacuum of space, but not UV. Only *Chroococcidiopsis* survived being exposed to full UV. Experiments by de la Torre *et al.* reported the outcomes of lichens, cyanobacteria and bacteria that were exposed to space in their native rock environment outside the international space station for 10 days.[29] The samples had been obtained from the Atacama Desert in Chile. The researchers found that the bacteria survived exposure to space, but that fewer survived when they were exposed to the full range of solar radiation. This study was focused on lithopanspermia, which is the theory that life was transferred to Earth via a meteorite originating on Mars or elsewhere.

Horneck et al. looked at the exposure of *Bacillus subtilis* spores to space.[30] On Spacelab 1, *B. subtilis* spores were exposed to vacuum and/or UV. Some were exposed to the full UV

spectrum, while others were exposed to just portions of the spectrum. Exposure to vacuum reduced survivability by 50%. In other work, Horneck et al. reported the survival of *Bacillus subtilis* spores in varied space conditions on different spacecraft missions.[31] As long as the spores were protected from the worst of the UV radiation, they could survive several years in space. They found that the spores are more sensitive to UV radiation when under vacuum. Additionally, they found that the structure of the spore DNA was changed because of the desiccating conditions in space, which led to more frequent mutations.

In another paper exploring panspermia, Horneck et al. report the outcome of monolayers and multilayers of *B. subtilis* that were left on board the NASA Long Duration Exposure Facility (LDEF) for almost six years.[32] In these experiments, spores survived the vacuum of space. Some of them also survived UV radiation, but the survival rate was much lower when they were exposed to UV. Without exposure to UV, up to 80% of the spores survived exposure to space. When they were also exposed to UV, survival was reduced by four orders of magnitude. Glucose and buffer salts helped protect the spores against vacuum conditions.

Wassmann et al. reported the outcome of endospores of *B. subtilis* that were exposed to conditions that simulated the surface of Mars for 559 days outside the International Space Station in the EXPOSE-E facility.[33] The spores survived when exposed to the conditions, but those who had less exposure to UV had a higher survival rate.

1.3.2 Previous Impact Studies

In addition to studies looking at microorganism survivability of space conditions, a few experiments testing microorganism survivability of extreme shock or acceleration have been

done. However, they were mostly done from a lithopanspermia perspective and aimed at demonstrating survivability under conditions simulating the interior of a meteorite.[34-37] These studies show widely variable survivability rates for different bacteria.[36] Fajardo-Cavazos et al. used ballistics experiments to test the impact survivability of *Bacillus subtilis* spores in the context of lithopanspermia.[38] They concluded that the spores could withstand impacts that had the kind of shock acceleration they would have experienced if ejected from Mars.

Horneck et al. exposed dry layers of *B. subtilis* spores to 5-50 GPa of shock to simulate the pressures found in Martian meteorites.[39] The layers were put between Martian rock simulants called gabbro discs. Spore survival as a function of pressure experienced was measured. Survival decreased as shock pressure increased, but the spores survived up to 41.5 GPa.

Mastrapa et al. tested *B. subtilis* survivability of high acceleration by centrifuging the spores in an ultracentrifuge and also by firing the bacteria with a rifle onto a target.[36] The spores experienced 4.27×10^6 m/s^2 acceleration in the ultracentrifuge. After 65 hours of centrifuging, 90% of the spore population was inactivated. In the rifle test, the rear cavities of lead pellets were filled with the spores. The lead pellets were fired from a compressed-air pellet rifle onto a plasticine target. They experienced a velocity of 100 m/s and an acceleration of 1.5×10^6 m/s^2 from one rifle and a velocity of 300 m/s and an acceleration of 4.5×10^6 m/s^2 from the other. Between 40-90% of the *B. subtilis* spores survived the ballistic tests.

Roten et al. also performed some ballistics experiments to test impact survival of *B. subtilis*.[34] They used a GP11 bullet that undergoes 100,000 G acceleration in half a millisecond. The bacteria survived the force but they didn't get quantitative results from the GP11 bullet. To

get quantitative results, they did pellet experiments in which the bullet with bacteria on it was completely stopped by a sterile target. In order to completely stop the bullet with the sterile target, they had to use less powerful guns. The *B. subtilis* spores showed significant survival of the acceleration. Burchell et al. loaded *B. subtilis* spores into a ceramic porous projectile in a light gas gun and fired at agar or ice.[40] They were found to survive up to 78 GPa.

Even with these experiments, there is a need for future impact work. The experiments were mostly shock and acceleration, rather than pure impact experiments. These experiments were also very costly and time-consuming. Electrospray ionization coupled with an acceleration device is a much less expensive alternative, costing almost nothing per experiment once the equipment is constructed and the method working. In order to use ESI for this purpose, however, the microorganisms must survive the process.

1.4 Other Possible Applications of Electrospraying Bacteria

In addition to enabling testing of impact survivability of bacteria, knowing whether bacteria survive electrospray could be useful for bacterial characterization with mass spectrometry, ion mobility spectrometry, or gas phase spectroscopy. If bacteria survive the electrospray process, then any of those techniques using a nondestructive detection method could be coupled with traditional biological characterization techniques that require live bacteria, such as culturing. Electrospray of bacteria could also be used for preparative bacterial arrays. If bacteria survive electrospray, then it would be possible to separate them based on their mass-to-charge ratio. A mixture of two or more species that have different enough ranges of mass to

charge ratios could be separated into an array. Then culturing or other analyses could be performed on the individual species. These experiments are also useful from a fundamental standpoint just to see if bacteria survive electrospray and impacts.

1.5 Experimental Overview

Electrospray survivability of both *Escheria coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis)* spores was tested first with atmospheric mobility experiments, and then *B. subtilis* electrospray survivability was also tested under vacuum conditions. The aim of the atmospheric mobility experiments was to see if bacteria could survive electrospray charging while desolvated and not agglomerated. The experiments were designed to collect only bacteria cells that met each of those criteria.

The atmospheric mobility experiments used the concept of mobility coefficient, which is also used in ion mobility spectrometry. In traditional ion mobility spectrometry (IMS), a group of ions is released together by a shutter.[41] After drifting through an electric field, they are detected at different times. The time it takes each ion to reach the end is based on its mobility coefficient. This mobility coefficient is determined by both experimental conditions and ion characteristics, including mass, charge, and cross sectional area. The mobility coefficient is larger for a particle with a smaller mass or cross sectional area and a greater number of charges.

Detection of viable bacteria in the atmospheric mobility experiments is different from detection in conventional IMS. In IMS the drift time is measured to identify which species of bacteria are present. Rather than trying to identify different species of bacteria, we were trying to see whether bacteria survived electrospray. We were only interested in detecting living bacteria.

Therefore, instead of measuring the charge hitting a detector, bacteria were cultured and counted to determine viability. Because culturing requires overnight incubation, it was impractical to do time resolved measurements. Therefore, in each experiment a continuous stream of charged bacteria from ESI entered the drift tube instead of a pulsed sample. This resulted in integrated data, because any bacteria that made it to the end under those conditions were measured.

The final technique used in these experiments is electrostatic deflection in vacuum. Electrostatic deflection is based on the force applied by an electric field on a charged particle. In electrostatic deflection, a beam of charged particles passes between two plates. The plates have a potential applied between them and the electric field from the potential deflects the charged particles. The particles are deflected different amounts depending on their mass to charge ratio. In these experiments, this technique is used to separate charged spores in space.

Herein, we present experimental results that conclusively show that *B. subtilis* spores remain viable after becoming electrically charged and desolvated using ESI. This could lead to enhanced capabilities in ion mobility spectrometry or mass spectrometry detection of bacterial species by coupling with other biological techniques, controlled selection and deposition of different bacteria species into arrays, and other applications such as providing the means to accelerate bacteria for impact studies.

2 ATMOSPHERIC MOBILITY EXPERIMENTS

2.1 *E. coli* Atmospheric Mobility Experiments

2.1.1 Experimental

Ampicillin resistant strains, L99A and M1060 of *E. coli* were suspended in nutrient broth for the experiments. Petri dishes containing 50μg/mL ampicillin were used for culturing. If strains that were not antibiotic resistant and normal petri dishes were used, then the entire environment of the experiment would have to be completely sterilized. That would be impractical. Using ampicillin resistant *E. coli* and ampicillin containing petri dishes ensures that the only bacteria grown were the *E. coli* we were using. Thus, we only had to ensure that there was no *E. coli* on the instrument before each experiment.

As described in the introduction, the aim of the atmospheric mobility experiments was to determine whether the species we are testing survive electrospray. To accomplish this, we built an instrument to test the survivability at atmospheric pressure. The instrument was designed so that neutral bacteria could be excluded. It was also designed with the ability to vary conditions to explore the range of charges that surviving bacteria have. A diagram of the instrumentation is shown in Figure 4. A picture of the instrumentation is shown in Figure 5.

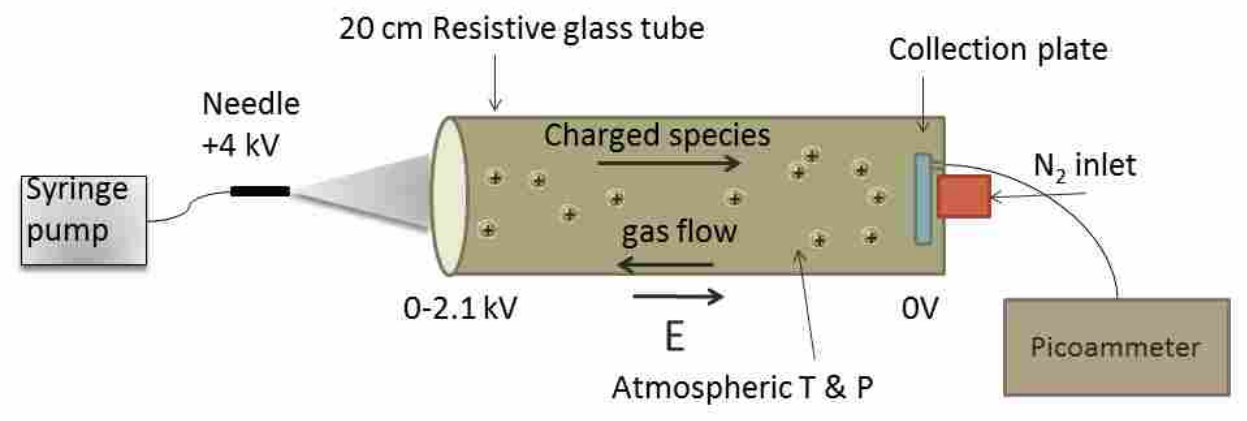

Figure 4. An illustration of the instrumentation used for the *E. coli* atmospheric mobility experiments.

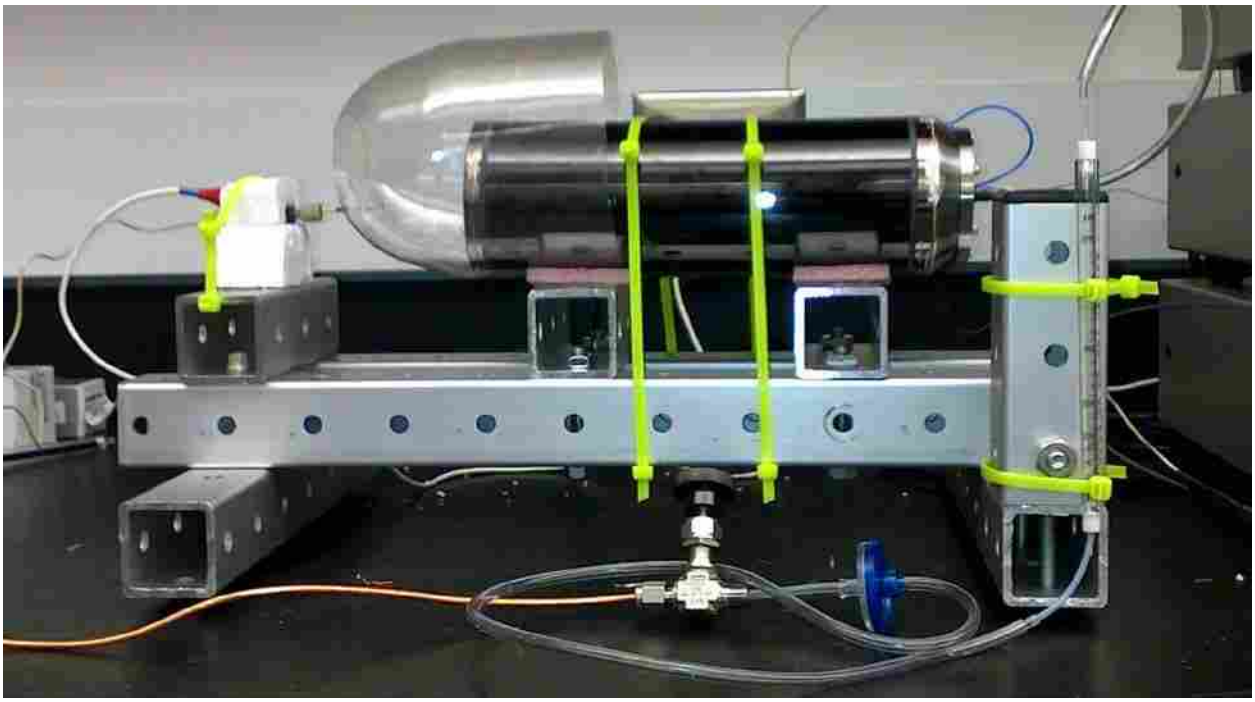

Figure 5. A picture of the instrumentation used for the *E. coli* atmospheric mobility experiments.

The instrumentation consisted of a KD Scientific syringe pump with a Hamilton 500 µL syringe connected by 1/16 inch PEEK tubing and Upchurch Fingertight PEEK fittings to an electrospray needle made with Small Parts 32-gauge stainless steel tubing. A Stanford Research Systems High Voltage Power Supply was connected to the electrospray needle. A 20 cm Fieldmaster doped lead silicate glass tube with 20 megaohms resistance was used as the drift tube after the electrospray needle. The front of the drift tube was connected to another Stanford Research Systems High Voltage Power Supply. Tungsten wire mesh covered the inlet of the drift tube so that the applied voltage was uniform across the whole inlet. A stainless steel 1 ¼ inch diameter collection plate was secured to the back of the drift tube and was grounded. A picture of the collection plate is shown in Figure 6. Dry nitrogen gas from a tank of nitrogen was connected to an inlet in the back of the drift tube and was able to flow around the collection plate. Gas flow rate was determined by a pressure regulator and a flow meter. Gas flow rate was monitored with a Gilmont Instruments Compact Flowmeter size #13. A Keithley 6485 picoammeter was connected to the collection plate to monitor the current from the spray.

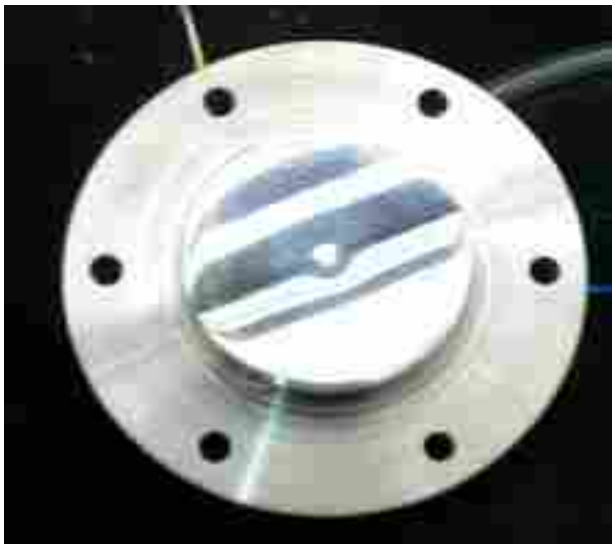

Figure 6. Collection plate.

Initially the setup was tested with solutions of polystyrene spheres. Those tests were used to ensure that the electrospray setup worked. Current measured with the picoammeter was used to verify that the electrosprayed particles were hitting the back of the tube. After initial testing of the instrument, *E. coli* was the first species used in the atmospheric mobility experiments. Some initial experiments were done which consisted of electrospraying the *E. coli* suspension directly onto the grounded metal collection plate without the drift tube in between. In these experiments, the collection plate was about an inch away from the electrospray needle. These were done to see if these strains of *E. coli* could survive the electrospray process under conditions similar to the experiments of Kim *et al*.[42] Some drying control experiments were performed by pipetting *E. coli* solution onto the collection plate and letting it dry. Then the plate was washed with sterile water and plated on a petri dish.

In the main experiments, suspensions of *E. coli* in nutrient broth were introduced via electrospray into the inlet of the drift tube. A large positive voltage, typically 4000-5000V, was

applied to the electrospray needle. From the needle, they were electrosprayed into the inlet of the drift tube. A positive voltage lower than the voltage applied to the electrospray needle, typically around 2500-3000 V, was applied to the front of the drift tube while the back of the drift tube was grounded. This created an electric field across the drift tube that drew electrically-charged species through the tube. There was also a countercurrent of dry nitrogen flowing in the opposite direction that the charged particles were drawn by the electric field. The nitrogen counter gas prevented neutral bacteria from reaching the end of the drift tube. Charged species traversed the drift region and were collected on a metal collection plate. The collection plate was sterilized with methanol before each experiment. It was swabbed and the swab was plated on a petri dish containing ampicillin to ensure that there was no *E. coli* on the plate before running the experiment. After each experiment, the contents of the collection plate were washed onto a petri dish. The plate was washed by pipetting 300 μL of sterile water onto it, swirling the water around, and then transferring the water with a pipette onto the petri dish. The sample was spread on the petri dish with a sterile spreader. The petri dish was cultured overnight at 39 °C. A picture of a test with positive results is shown in Figure 7. In all of the experiments the electrospray voltage, the voltage applied to the front of the tube, and the flow rate of the bacteria solution could be adjusted. To ensure that the *E. coli* could survive drying on the collection plate, some of the electrospray solution was pipetted onto the collection plate. It was left to dry and then the collection plate was swabbed and cultured to look for growth. To test if the *E. coli* was not sticking to the drift tube, the drift tube was sterilized before an experiment and then swabbed and cultured afterward to look for growth.

Figure 7. Positive results from an *E. coli* test.

2.1.2 Results and Discussion

E. coli did survive the preliminary experiments where it was electrosprayed directly onto the collection plates. In those experiments, it was evident that the *E. coli* was not desolvated. There was visible pooling of liquid on the collection plate. These experiments replicated some of the conditions in the experiments by Kim et al.[15] Although the *E. coli* was aerosolized with electrospray, it was not desolvated and thus the individual *E. coli* cells were not charged. *E. coli* also survived the drying control, which indicates that they can survive drying in the absence of an electric field.

In all of the experiments, the current reading from the picoammeter indicated whether the electrospray was hitting the collection plate, but it did not give any information about the

analyte. The background current from the spray was much larger than the current from the analyte. The current was reduced somewhat with increased counter-gas flow, indicating that at high gas flow fewer particles reached the end. It was very noisy though, and thus difficult to measure. There was not a significant change in current when altering the liquid flow rate. This may be because at higher flow rates the electrospray process became less efficient and a smaller relative fraction of particles was ionized.

Table 1. This shows measured current as a function of of altering the gas flow. These tests were performed with a 0.005% w/v 2.19 µm polystyrene solution in 4:1 MeOH: H_2O modified to pH 3.9 with formic acid.

Gas Flow (L/min)	Current (nA)
0-5	0.14-0.15
6	0.13-0.14
7	0.12-0.13
8.5	0.13

E. coli inconsistently survived the atmospheric mobility experiments where it was electrosprayed through the drift tube. In those, the *E. coli* sometimes survived, but what looked like dried water droplets were visible on the plate. When dried water droplets were not visible, the *E. coli* did not survive. This implies that *E. coli* only survived electrospray when it was still solvated at the time the droplets contacted the collection plate and were neutralized. Because *E. coli* survived the drying control, we can infer that it was the charging of the cells that killed

them. From this we can conclude the *E. coli* does not survive all of the conditions of electrospray.

2.2 *B. subtilis* Atmospheric Mobility Experiments

2.2.1 Experimental

Because *E. coli* did not survive, we moved onto another species that was hardier, *Bacillus subtilis*. *B. subtilis* strain 1A308, which is resistant to rifampicin, was used in these experiments. A rifampicin resistant strain was used to prevent false positives. All petri dishes used to culture *B. subtilis* in this experiment consisted of nutrient agar with 2 μg/mL rifampicin to ensure that only the *B. subtilis* we used in the experiment would grow on them. The *B. subtilis* was sporulated according to standard procedures.[43] The spores were then harvested and suspended in water. The concentration of the spores was determined through serial dilutions. A small known volume of each serial dilution was cultured on a petri dish. The cultured cells were counted and multiplied by the dilution factor to determine the concentration of the original solution.

Figure 8. A picture of the instrument used for *B. subtilis* atmospheric mobility experiments.

The same instrument used in the *E. coli* experiments was used for the *B. subtilis* experiments, but additional modifications were made to the setup. A picture of the instrument is shown in Figure 8. As before, the instrumentation consisted of a KD Scientific syringe pump with a Hamilton 500 μL syringe connected by 1/16 inch PEEK tubing and Upchurch Fingertight PEEK fittings to an electrospray needle made with Small Parts 32-gauge stainless steel tubing. A Stanford Research Systems High Voltage Power Supply was connected to the electrospray needle. A 20 cm Fieldmaster doped lead silicate glass tube with 20 megaohms resistance was used as the drift tube after the electrospray needle. Tungsten wire mesh covered the inlet of the drift tube so that the applied voltage was uniform across the whole inlet. A stainless steel collection plate was secured to the back of the drift tube. Dry nitrogen gas was connected to an inlet in the back of the drift tube and was able to flow around the collection plate. A diagram of the instrument is shown in Figure 9.

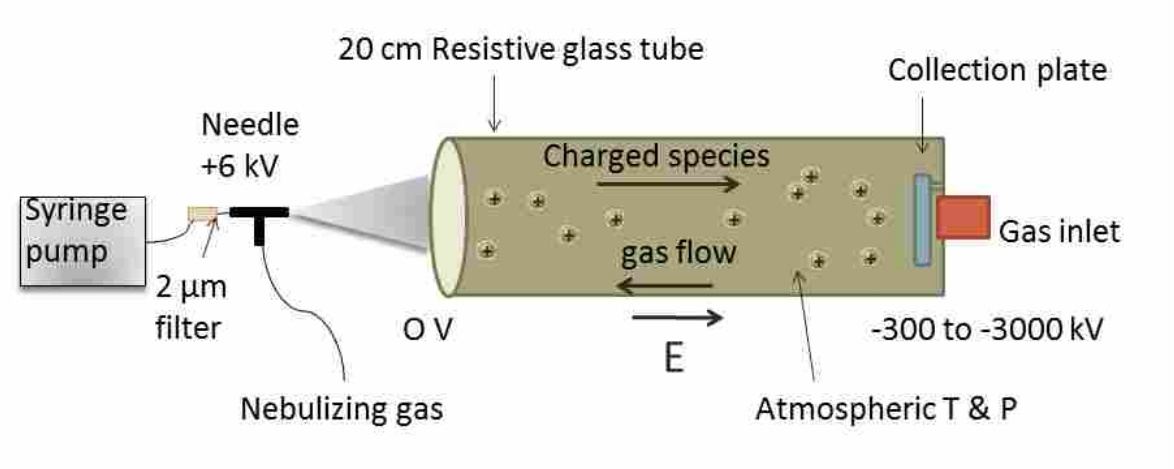

Figure 9. A diagram of the instrument setup used for the *B. subtilis* experiments.

For these experiments, dry nitrogen nebulizing gas flowing around the electrospray needle was added to aid desolvation. The N_2 nebulizing gas was set up with an Upchurch P727 Tee. The small, 32-gauge electrospray needle went straight through the Tee connection with the gas flowing around it as shown in Figure 10. A picture of the electrospray needle is shown in Figure 11. Both the counter-gas and the nebulizing gas flow rates were monitored with a Gilmont Instruments Compact Flowmeter size #13. A 2 µm Upchurch Scientific Frit-In-A-Ferrule was placed immediately before the electrospray needle to exclude agglomerated bacteria.

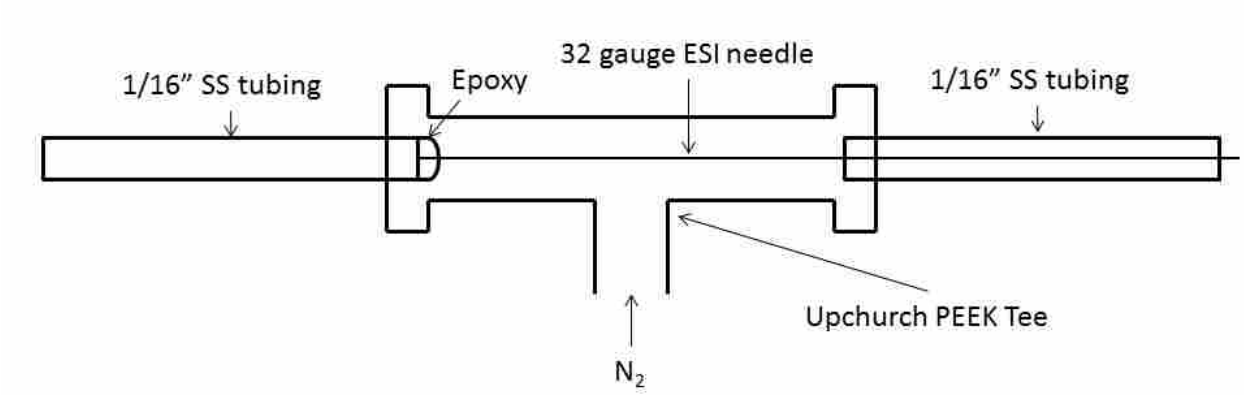

Figure 10. Diagram of the electrospray needle with a tee for nebulizing gas.

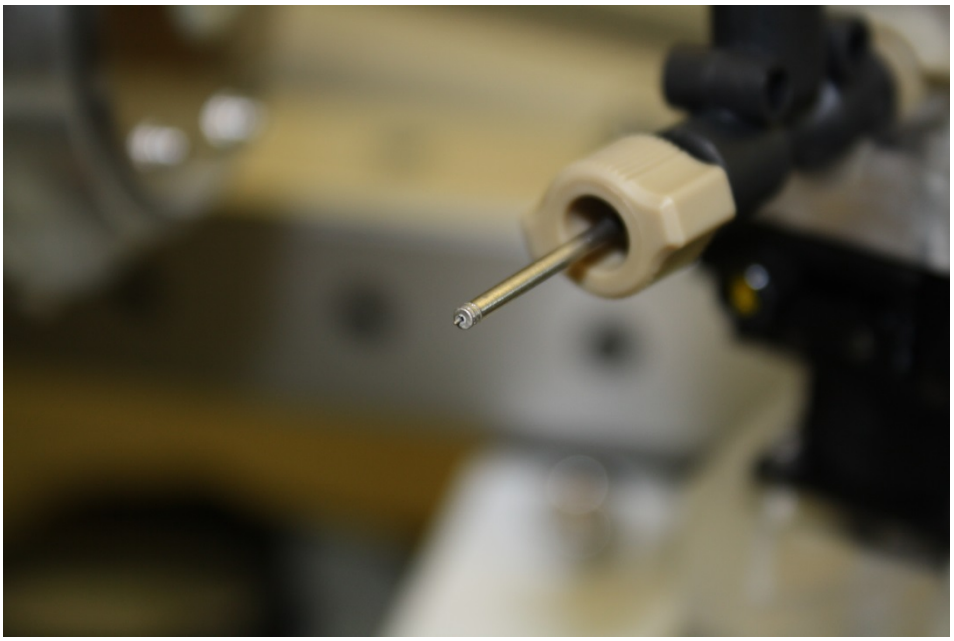

Figure 11. Picture of the electrospray needle with the nebulizing gas setup.

In the first experiments with *B. subtilis*, the voltage was applied in the same way that it was in the *E. coli* experiments (the front of the tube was at a lower positive voltage than the electrospray needle and the back of the tube was grounded). Later, to get a bigger potential across the tube, the front of the drift tube was grounded and a constant negative voltage (that could be set between -1000 to -5000 V) was applied to the back end of the drift tube. In the experiments we could adjust the electrospray voltage, the voltage applied to the tube, the flow rate of the nebulizing gas, the flow rate of the counter gas (up to 0.3 m/s), and the flow rate of the *B. subtilis* solution.

Before each trial, the collection plate was sterilized with bleach. The plate was swabbed and cultured to ensure that it was sterile. Suspensions of *B. subtilis* in sterile water were introduced via electrospray into the inlet of the drift tube. After each trial, the contents of the

collection plate were washed onto a petri dish with rifampicin-containing agar with 300 µl of water. The method of washing was the same as in the *E. coli* experiments. The water was pipetted onto the plate, swirled around, and then transferred via pipette to the petri dish. The sample was spread on the petri dish with a sterile spreader. After overnight incubation at 39°C, the colonies were counted. The concentration of the spore solution was previously determined by counting the colonies from cultures of serial dilution. Typically a solution with a concentration of 66000 colony forming units (spores) per milliliter was used. Percent survivability was determined by comparing the live counts from the culture of the collection plate to the number of spores contained in the solution that was electrosprayed into the drift tube. Negative controls were run with the gas in the absence of bacteria to ensure that the nitrogen was not contaminated. Sterilization controls were also performed.

2.2.2 Results and Discussion

The design for the atmospheric mobility experiments ensured that any bacteria detected at the end of the tube must have been charged, desolvated and de-agglomerated. The dry nitrogen nebulizing gas and counter gas ensured that the bacteria were desolvated. The nitrogen counter gas and voltage applied to the drift tube ensured that the bacteria which were collected at the end were charged. The filter in the electrospray line ensured that the bacteria were not agglomerated. Therefore, any bacteria that were cultured from the collection plate were charged, desolvated and de-agglomerated.

In these experiments, we never observed dried water droplets on the collection plate or any other sign that the solvent was reaching the plate. This is likely due to the addition of the

nebulizing gas. We had tested the electrospray tip with the nebulizing gas for some of the last *E. coli* experiments, but the gas system was not set up to allow both nebulizing gas and counter gas at the same time until the *B. subtilis* experiments. Unlike *E. coli*, *B. subtilis* spores did survive desolvation and charging at atmospheric pressure. The difference in survival is likely due to the difference between the two types of cells. *E. coli* is gram negative and does not form spores, while *B. subtilis* is gram positive and does form spores. Gram positive and gram negative bacteria have different membrane structure, but because the *B. subtilis* was sporulated, the most important factor in the survival of the *B. subtilis* spores was likely their spore coat. Other research into *B. subtilis* extremophile behavior has shown the spore coat to be key in *B. subtilis* survival of harsh conditions.[20] Further experiments done with unsporulated *B. subtilis*, other *Bacillus* spores, and other gram negative and gram positive bacteria are necessary to further explore the factors behind survivability.

The recovery rate of *B. subtilis* spores was less than 20% at atmospheric pressure, but the survival rate is likely higher. There are a few reasons that not all of the spores in the solution would have made it to the end of the drift tube. In electrospray, not every analyte becomes ionized.[44] Therefore, not all of the bacteria in the solution would be charged. Additionally, some would have been lost before they reached the drift tube.

It was important to ensure that the bacteria were de-agglomerated and desolvated. Bacteria are prone to agglomeration.[45] If the electrosprayed bacteria were still agglomerated, the bacteria on the inside of the agglomerate would not have become charged. Thus the bacteria that were cultured wouldn't necessarily be charged. With the filter used in this experiment, the *B. subtilis* could have potentially gone through in pairs if they were exactly aligned. Still, both

bacteria would still be charged because they would both be on the surface. Desolvation is important for a similar reason. If the spore was surrounded by water, then it would not experience an electric field because the charges would all migrate to the surface of the water droplet. Thus the bacteria that survived would not be verified as actually charged themselves. Because of the steps taken to ensure desolvation and de-agglomeration in these experiments, the *B. subtilis* spores that survived were definitely charged themselves.

We expected a distribution of mobility coefficients due to statistical ESI charging and differing shape and size among bacteria spores.[2, 24, 25] In an attempt to measure this distribution, we ran experiments at different drift tube voltages. In our experiment, because of how we measure, each data point at a given voltage should include all spores with a certain charge or above. Those spores with too low of a charge would be knocked out by the drift gas because the force of the electric field on those spores would not be large enough to overcome the force of the drift gas pushing them out of the tube. With a larger potential across the tube, the force of the electric field pulling the charged bacteria through is proportionally larger. Therefore, with a bigger voltage across the tube, the spores would not require as high a charge to get enough force from the electric field to overcome the force of the counter-gas pushing them out. Thus, at low drift tube voltages the spores that make it to the end would have a high charge state, and at higher drift tube voltages, both spores that are highly charged and those with less charge would make it through. Because our data is integrated and the charge (and thus the mobility coefficients) should have a Gaussian distribution, we expected our data to be an s-curve, the integral of a Gaussian. This expected outcome is demonstrated in Figure 12.

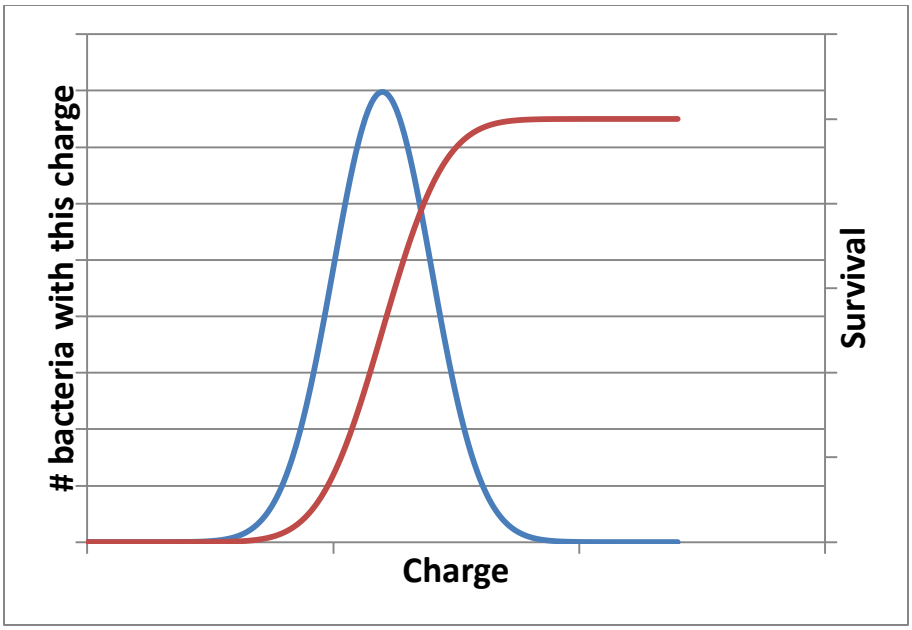

Figure 12. Expected charge distribution and the expected form of the integrated data.

Initially, the data from the experiments with different drift tube voltages looked as expected. The data is shown in Figure 13. It followed the beginning of an S-curve as shown in Figure 14. We hypothesized that we just needed to test higher voltages to get the rest of the curve. The 5000 V power supply was switched out for a power supply that could reach 7500V. After testing the higher voltages (not all the way up to 7500V because of a resistor put in the electrical line connecting the tube and the power supply for safety reasons) the curve still looked the same. Based on the work of Fuerstenau *et al*., we expected the spores to have between $7x10^4$ and $2x10^5$ charges.[12] This range was calculated by taking the number of charges per surface area of the electrosprayed Tobacco Mosaic Virus, which is rod-shaped, and then extrapolating up for the surface area of the *B. subtilis* spores, which are also rod-shaped. Both were assumed to have cylindrical surface areas. The range of dimensions used for *B. subtilis* was the range given in section 1.2. The original calculations for the charge on the surviving spores were based on

balancing the drag force with the force of the electric field. These calculations predicted that at those charge levels we should have observed an S-curve. We did some new calculations on the charges necessary for the spores to traverse the drift tube using a momentum mean free path. From those calculations we found that the charge the spores needed was well below what it was expected that they had. That combined with the fact that we were not getting more of the S-curve demonstrated that some other phenomenon must be causing the trend in our data.

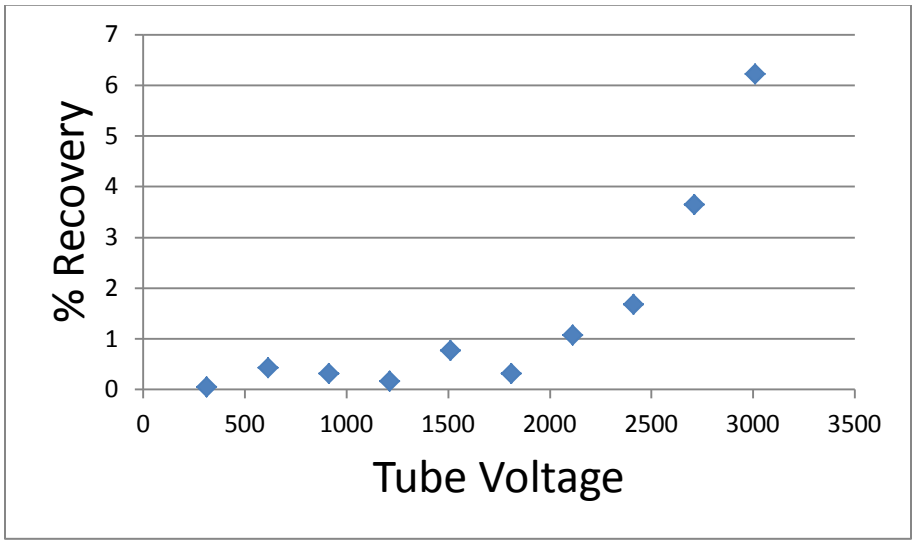

Figure 13. Data showing the % recovery of B. subtilis versus the tube voltage.

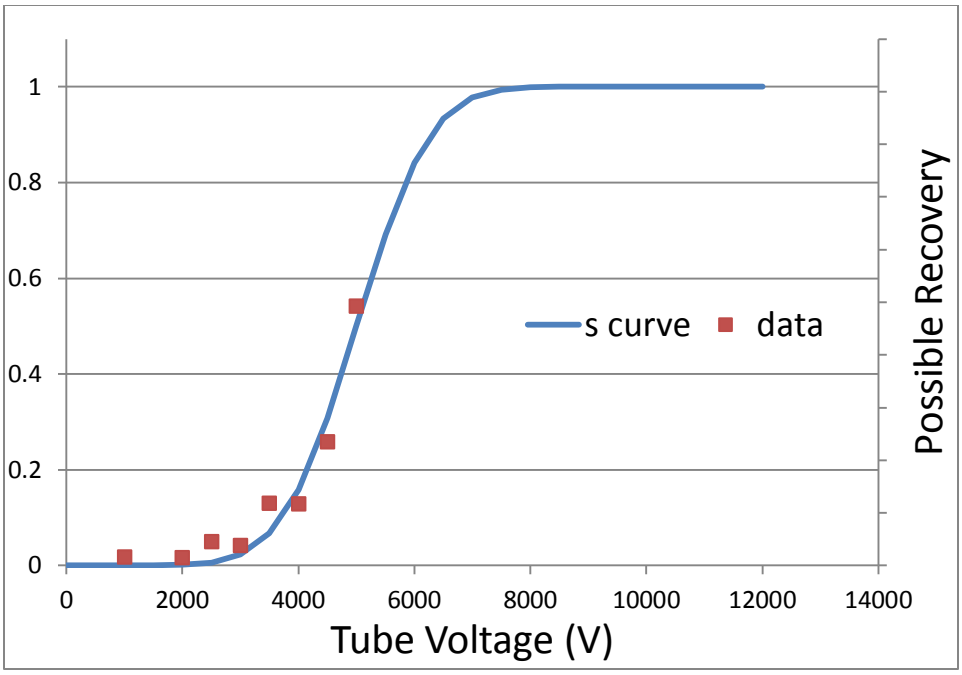

Figure 14. Curve overlaid on data of B. subtilis % recovery versus tube voltage.

Up to that point, the experiments were performed each day starting with collecting data at the highest tube voltage and then collecting data for subsequently lower voltages. Because the data was collected in the same order each day, we thought that perhaps time was the variable which was causing the observed trend. We did more experiments, this time starting with a low voltage and going to a high voltage. We also ran experiments taking data for different voltages randomly. The results from both types of experiments showed the same trend. The percent recovery correlated to the order that the experiment was run, as shown in Figure 15. The experiments that were run earlier in the day had higher recovery rates than the experiments that were run later in the day. This trend was consistent for all voltage orders. This is likely due to settling of the spores out of suspension. In the end, it was found that the percent recovery of the bacteria did not change noticeably with different voltages or nitrogen counter gas flow rate.

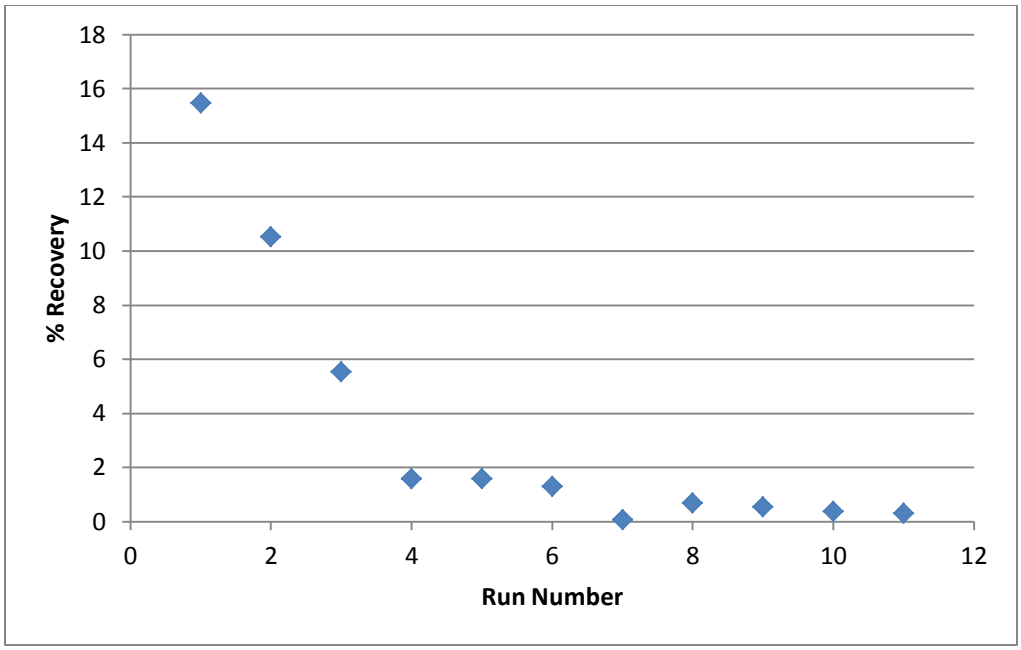

Figure 15. Figure illustrating the phenomenon of % recovery being dependent on run number.

Even though we were unable to get the charge distribution, this work addresses shortcomings of the previous work[15] of Kim et al. They studied the use of electrospray for viable bioaerosols with *Staphylococcus epidermidis* and *Escherichia coli*. They found that the bacteria survived, but they report the electrosprayed bacteria droplet size greater than the size of the bacteria. Therefore, we know that in their experiment the bacteria were either not desolvated or not de-agglomerated. There was no method applied to ensure the bacteria were not agglomerated besides relying on the repulsive columbic force. In their work, they reported that the bacteria survived electrospray, but the bacteria including *E. coli* was not desolvated, so they did not truly survive the complete process. They were only trying to use electrospray to form a bacterial aerosol, which they were able to do. However, there wasn't enough information to generalize

their findings of viability and apply it to future mass spectrometry studies. That is the area that our work addresses.

From the atmospheric mobility experiments, we observed that *E. coli* do not survive all electrospray conditions, but *B. subtilis* spores do. While we were able to demonstrate electrospray survivability of *B. subtilis* spores, we were unable to observe the charge distribution of the viable spores. Therefore, we needed further work to find the charge distribution.

3 VACUUM DEFLECTION EXPERIMENTS

3.1 Introduction

After discovering that *B. subtilis* spores survive electrospray under atmospheric conditions, we wanted to see if they survive electrospray and subsequent introduction into vacuum. Additionally, we wanted to see what charge distribution the viable spores had because we were unable to find that with the atmospheric experiments.

Another member of the group performed some experiments using an image charge detector to try and discover what charges the *B. subtilis* spores had when electrosprayed. The charge detector consisted of a metal tube connected to a charge-sensitive amplifier.[46] When a charged particle passes through the metal tube, it induces an image charge, which, after signal processing, appears as an up-peak and a down peak corresponding to when the particle enters and exits the tube.[47] The area of the peak corresponds to the charge of the particle and the time between peaks corresponds to the time the particle was in the charge detector.

The instrumentation for the charge detection experiments consisted of a needle electrospraying into a differentially pumped inlet into a vacuum system. Inside the system, the electrosprayed spores were collimated in a beam tube and passed through a series of skimmers and pressure differentials. In the final chamber, there was a charge detector with a collection vessel behind it. This instrument is shown in Figure 16. He was able to see a signal from the *B. subtilis* on the charge detector. He also collected viable *B. subtilis* from the collection vessel. With the way the experiments were designed, however, the bacteria that were collected were not

necessarily charged and the bacteria that produced the signal at the charge detector were not necessarily viable. Because everything went in a straight line, neutral bacteria could have potentially made it to the collection vessel.

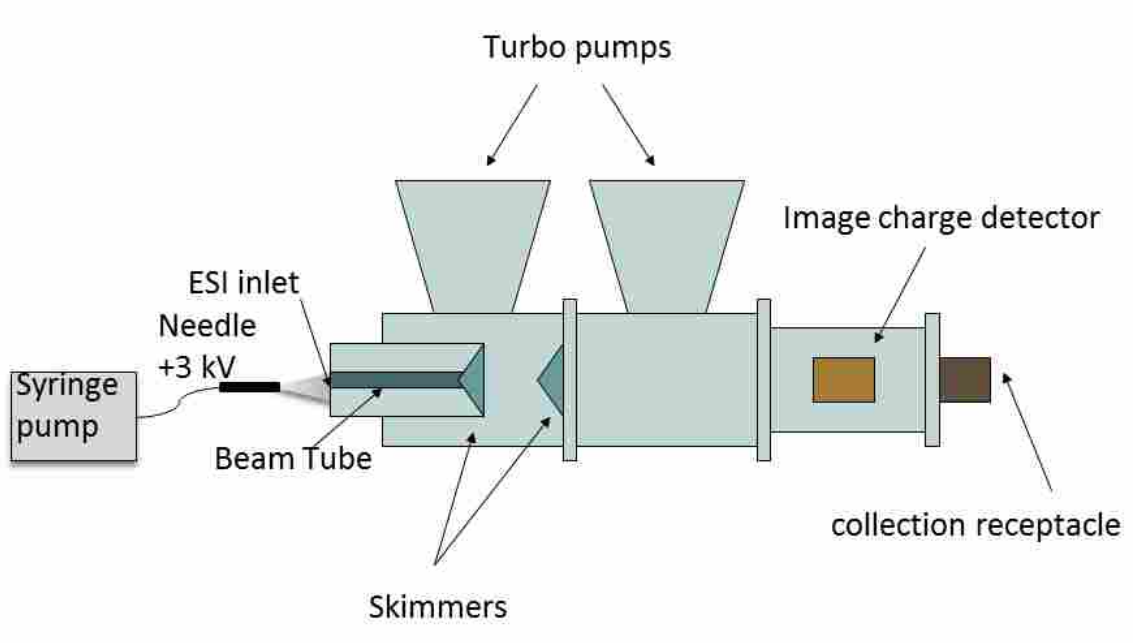

Figure 16. Instrumentation used in the charge detection experiments.

Also, the charge detection experiments were unable to give us the charge distribution. Based on the calibration done by another member of the group, the spores were measured with an amount of charge an order of magnitude larger than the Rayleigh limit. This is very improbable. Even if the calibration was correct, we could not get the full charge distribution because the charge detector needs many charges to measure signal. Therefore, we wouldn't be able to see any spores that weren't highly charged. Also, with this experimental setup we would not know if the charged bacteria were viable. Because of this we turned to another method to measure the charge distribution of viable spores.

The other method we turned to is electrostatic deflection. This is based on the principle[48] that a charged particle moving through an electric field experiences the force of the electric field: $F = qE$. That force accelerates the particle: $F = ma \rightarrow a_E = \frac{qE}{m}$. This acceleration is inversely proportional to the mass-to-charge ratio. Therefore particles with higher charge or lower mass will be deflected more strongly as shown in Figure 17.

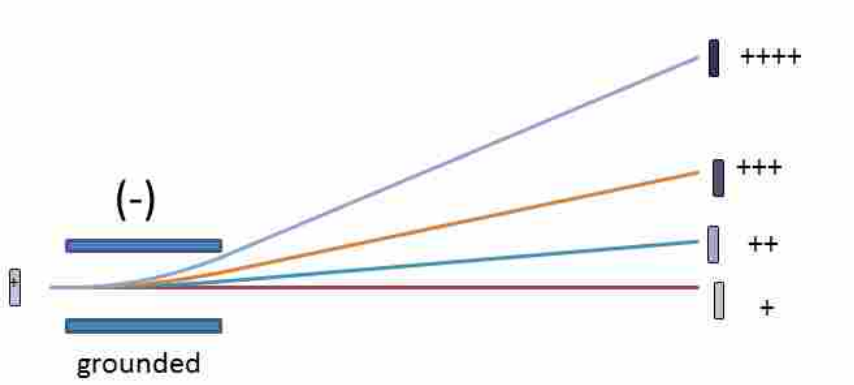

Figure 17. Diagram of the electrostatic deflection principle

In these experiments, the charged particles are spores. We designed a deflector where the electric field is created by two parallel stainless steel plates. We also designed a collector to be positioned after the deflector. This collector has different slots to collect bacteria with various mass to charge ratios depending on the electric field deflecting the spores. A picture of this collector and deflector, nicknamed the "Bug Trap" is shown in Figure 18. The initial vacuum deflection experiments were performed with positive electrospray. Later experiments were performed with negative electrospray (negative voltage applied to spray needle) to determine what impact the change in electrospraying bias had on the survival of the spores.

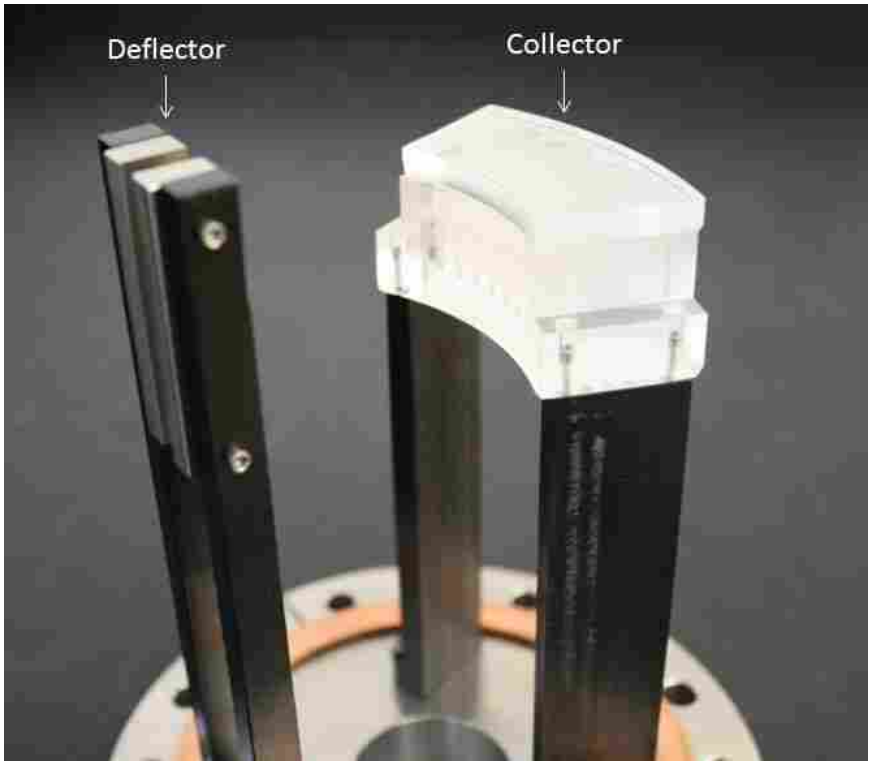

Figure 18. Picture of the deflector and collector.

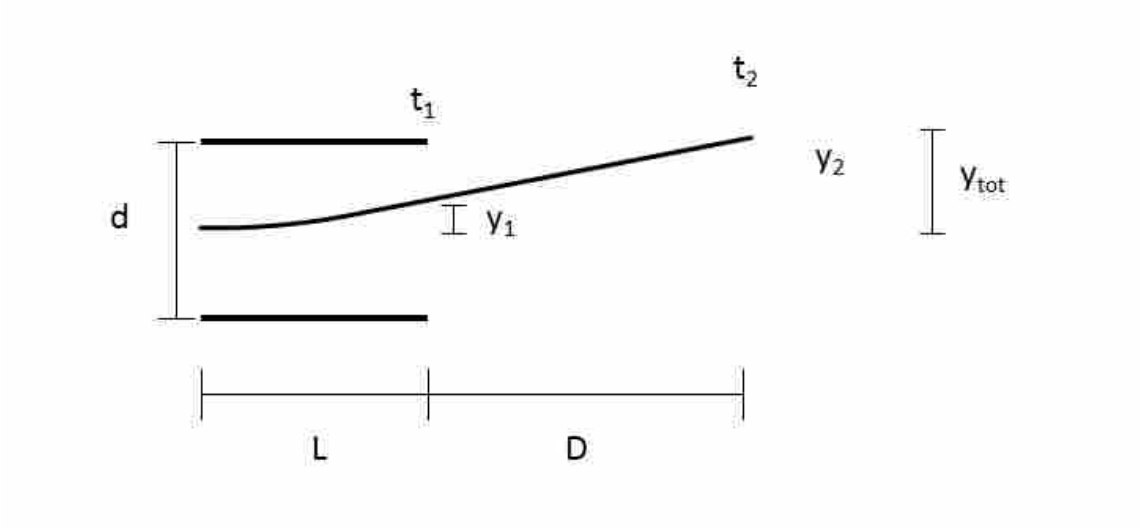

Figure 19. Diagram of the terms used in the electrostatic deflection calculations.

A diagram illustrating the variables in the equations is found in Figure 19. A more in-depth look at the theory and calculations behind electrostatic deflection follows: As mentioned before, the acceleration of a charged particle due to the electric field can be calculated from fundamental equations as

$$a_E = \frac{qE}{m},$$

where a_E is the acceleration due to the electric field, q is the charge of the particle in coulombs, m is the mass of the particle in kilograms, and E is the electric field in volts per meter. In the case of electrostatic deflection, this acceleration is in the y direction, so

$$a_y = \frac{qE}{m},$$

where a_y is the acceleration in the y direction. In the case of a voltage applied between two parallel plates, the electric field can be approximated as

$$E = \frac{V}{d},$$

where V is the potential between the two plates and d is the distance between them in meters. In this case, the acceleration in the y direction can then be expressed as

$$a_y = \frac{qV}{md}.$$

Because there is no initial velocity in the y direction, the distance in the y direction that the charged particle travels while between the stainless steel plates (y_1) can be calculated by

$$y_1 = \frac{1}{2} a_y t_1^2 ,$$

where t_1 is the time the particle spends between the plates. The variable t_1 can be calculated by the following equation

$$t_1 = \frac{L}{v_x},$$

where v_x is the velocity in the x direction in meters per second. In the case of these experiments, an average value of v_x was determined from the charge detection experiments. Substituting in the equation for t_1 yields

$$y_1 = \frac{qVL^2}{2mdv_x^2} .$$

To find the total distance that the charged particle travels, the distance it travels after exiting the deflection plates (y_2) must be calculated, so the total distance is found by

$$y_{tot} = y_1 + y_2 .$$

After the particle exits the deflection plate, it no longer experiences acceleration from the electric field, so y_2 can be calculated by

$$y_2 = v_y t_2 \ ,$$

where v_y is the y velocity of the particle when it exits the deflection plates. That is expressed as

$$v_y = a_y t_1 .$$

Substituting in the known expression for a_y yields

$$v_y = \frac{qVL}{mdv_x} \ .$$

The variable t_2 can be expressed as

$$t_2 = \frac{D}{v_x} \ ,$$

where D is the distance between the end of the deflection plates and the collector. After substituting in the expression for v_y and t_2 the following expression for y_2 is obtained:

$$y_2 = \frac{qVLD}{mdv_x^2} .$$

Adding both distances gives

$$y_{tot} = \frac{qVL^2}{2mdv_x^2} + \frac{qVLD}{mdv_x^2}$$

as the final expression for the total y distance the charged particle travels. Rearranging the equation to solve for charge yields:

$$q = \frac{y_{tot}}{\frac{1}{2}\frac{vL^2}{mdv_x{}^2} + \frac{vLD}{mdv_x^2}}$$

As stated before, q is the charge, y_{tot} is the total deflected distance in the y direction, v is the voltage, m is the mass of the particle, L is the distance from the deflector to the collector in the x

direction, D is the length of the deflector, and v_x is the velocity in the x direction. Because D<L, the second term in the denominator may be negligible.

3.2 Instrument Setup

The instrumentation for these experiments consisted of a KD Scientific syringe pump with a Hamilton 500 µL syringe connected by 1/16 inch PEEK tubing and Upchurch Fingertight™ PEEK fittings to an electrospray needle made with Small Parts 32-gauge stainless steel tubing. An SRS High Voltage Power Supply provided the voltage for the electrospray needle. This setup used the same electrospray needle used for the *B. subtilis* atmospheric modeling experiments. Again, a 2 µm filter was placed immediately before the electrospray needle to exclude agglomerated bacteria. The same Upchurch PEEK tee connection was used to supply dry nitrogen nebulizing gas that flowed around the electrospray needle to aid desolvation. The nebulizing gas flow rate was monitored with a Gilmont Instruments Compact Flowmeter size #13. The electrospray needle setup is shown in Figure 20.

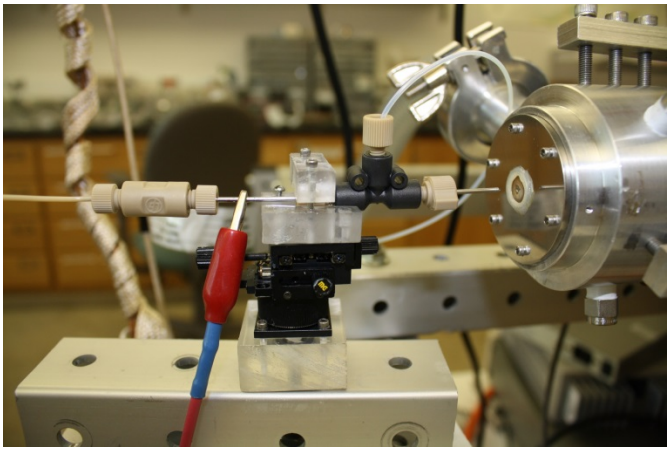

Figure 20. Picture of the electrospray needle setup for the vacuum deflection experiments.

In these experiments, the nitrogen nebulizing gas was heated by heat tape from Brisk Heat wrapped around the copper gas line. It was powered by a Staco Energy Products Variable Autotransformer. The gas flow was monitored with a flow meter. This heated nitrogen setup is shown in Figure 21. These experiments used the same vacuum system that had previously been used for the charge detector experiments. The vacuum system consists of 3 stages of differentially pumped vacuum systems. The second two stages are pumped with turbo pumps. The final stage has a pressure in the 10^{-5} torr range. At first, an aerodynamic lens was used to collimate the particle beam instead of the beam tube. In later experiments, the beam tube was put back in because the aerodynamic lens did not work. The end of the last chamber which contained the charge detector was removed because it was no longer needed. Instead, the collector and deflector, or Bug Trap, was placed after the beam tube and skimmer cones. A diagram of the entire vacuum setup is shown in Figure 22 and a picture of the setup is shown in Figure 24.

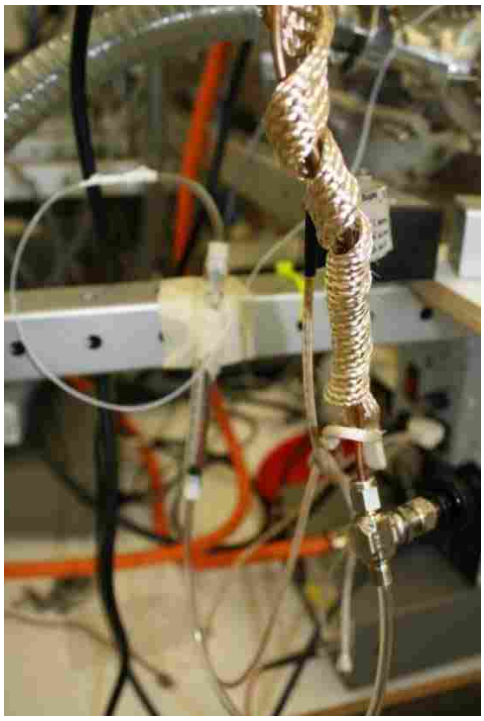

Figure 21. Heated nitrogen gas setup.

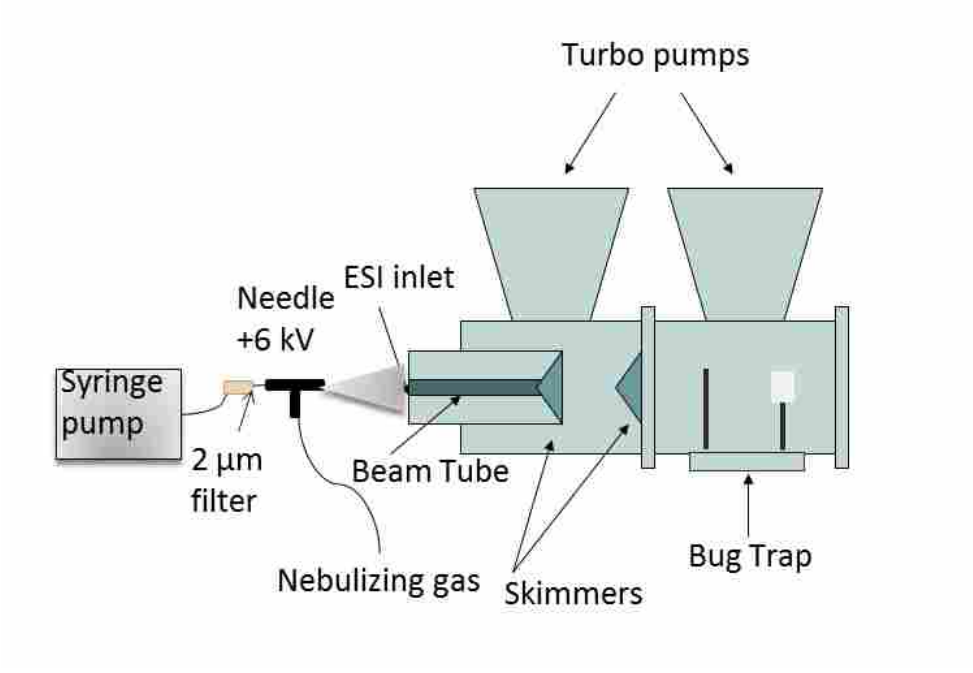

Figure 22. Diagram of the vacuum deflection instrumentation.

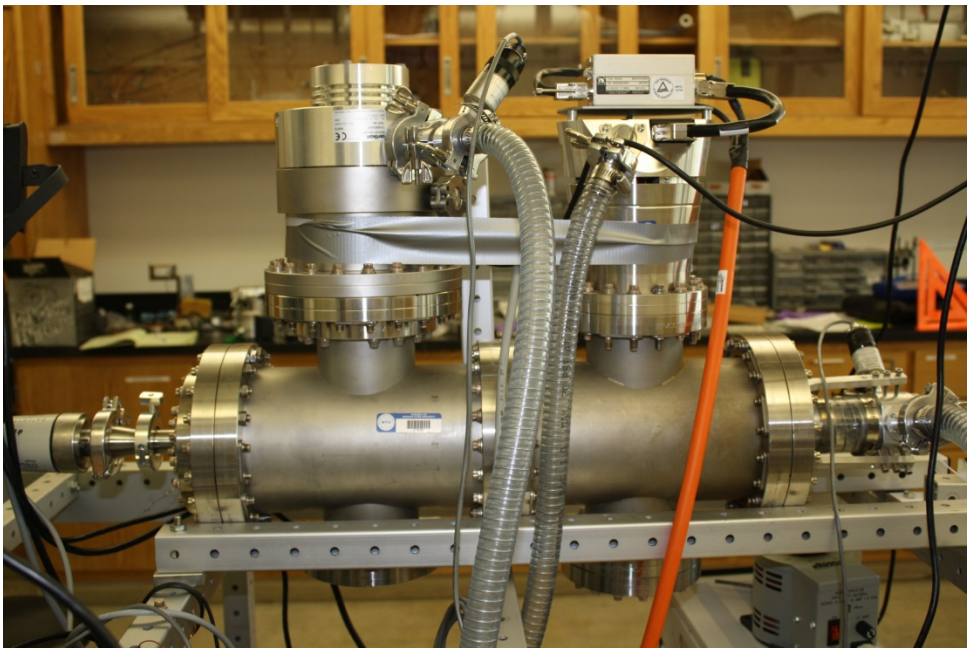

Figure 23. Picture of the vacuum deflection instrumentation.

The Bug Trap was mounted onto a flange so that it could be easily removed after each experiment. A drawing of the whole Bug Trap is shown in Figure 24. The deflector portion consists of two stainless steel metal plates on a Delrin stand. One of the plates was grounded and the other was connected to a power supply. For high voltages it was connected to a Stanford Research Systems High Voltage Power Supply and for low voltages it was connected to an Agilent Triple Output DC Power Supply. An acrylic collector on a Delrin stand with 7 slots was placed after the deflector plates. To better trap the spores, the back of the slots is deeper than the front. A detailed view of the collection channels is shown in Figure 25.

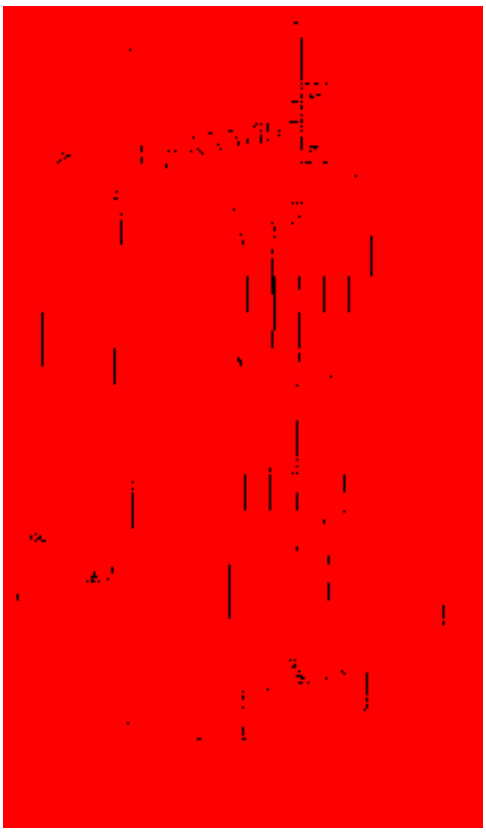

Figure 24. Design drawing of the Bug Trap.

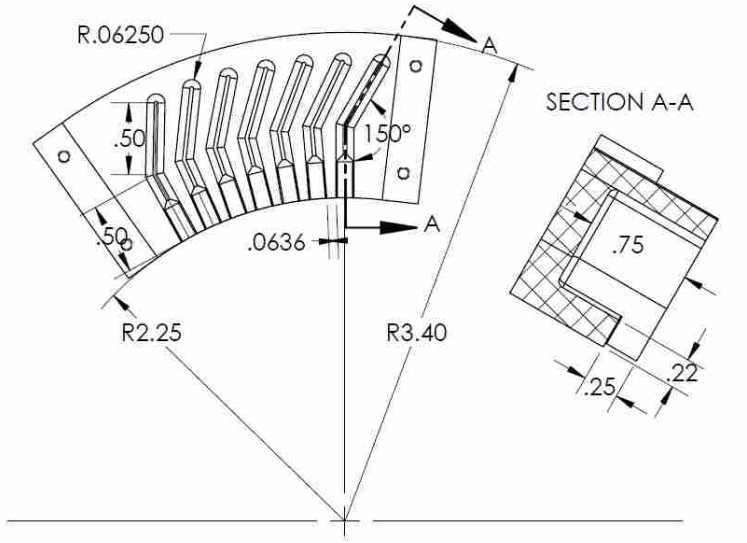

Figure 25. Detail of the collection channels.

After manufacture, the back corners of both the collector and the collector stands were belt sanded. This was necessary because the inside of the arm of the vacuum chamber was smaller than the interior diameter of the copper gasket, which is what the design dimensions were based on. The collector was originally designed to be aligned with the geometric center of the vacuum chamber, but in later experiments, to center the collector to the beam of spores, it was raised 3 mm higher with 5 #6 washers. It was assumed that channel #4 would collect the center of the beam, but it was later found that the beam of spores was centered on channel #3. At one point in troubleshooting the experiment, a flat 7 cm wide by 4.5 cm high Styrofoam collector was used to locate the particle beam. It had 10 labeled sections- 5 on top and 5 on bottom.

3.3 Experiments

The collector was washed out with and submerged overnight in a 10% bleach solution to sterilize it. Before each experiment, the collector was then washed out 3 times with sterile water to remove the bleach. Each slot was then swabbed and plated on a rifampicin containing petri dish to ensure that no *B. subtilis* was present before the experiment. A picture of a negative control is shown in Figure 26.

Figure 26. Petri dish for a deflection experiment negative control.

For each experiment, a suspension of *B. subtilis* spores in water was electrosprayed into the vacuum system. The settling problem was fixed for these experiments by using freshly vortexed solution for each experiment. The typical concentration of the spore solution was 660,000 colony forming units per milliliter. The nebulizing gas was set to 1 L/min. The electrospray voltage was set to give a stable electrospray, which occurred at 3700V when the needle was 1 cm from the inlet. The electrosprayed spores passed through the beam tube and skimmers and between the two metal plates. One of the plates was always grounded. The other plate was set at a voltage between 0 and 5000 V for each experiment. The spores landed in different slots on the collection vessel depending on the charge on the spore and the voltage applied to the plates. After each experiment, the flange containing the Bug Trap was removed from the vacuum system. The collector was detached and each slot was individually washed out with sterile water and plated on a rifampicin-containing nutrient agar petri dish. The washing procedure consisted of filling up a slot with sterile water using a sterile syringe. Then, the syringe was used to take the water back out of the slot and transfer it to the petri dish where the

water was spread with a sterile spreader. The petri dishes were incubated overnight at 39 °C. The next day, the colonies that had grown on the plates were counted. Experiments with both positive and negative electrospray were performed.

During the troubleshooting experiments with the Styrofoam collector, the procedures were the same except for sterilization and collection. The Styrofoam collector was wiped with KimWipes soaked in bleach to sterilize it. It was swabbed and plated before each experiment to ensure that it was sterilized. After each experiment, a sterile cotton swab dipped in sterile water was used to swab and plate each section to see where the bacteria landed. The instrumentation was the same except that in the early troubleshooting experiments the aerodynamic lens was used instead of the beam tube. Also, for some experiments, the deflector plates were removed. In all of these experiments, the Styrofoam collector was placed on the Delrin stand instead of the original collector.

3.4 Results and Discussion

During initial experiments, no bacteria were cultured out of any of the slots in the collector. To troubleshoot, a Styrofoam collector replaced the acrylic collector. This allowed a greater surface area for collection. At first, no bacteria were collected on it. The aerodynamic lens was switched out for the beam tube. This resulted in bacteria being collected on the top portion of the Styrofoam collector. The aerodynamic lens had been designed so that particles in the complete size range of *B. subtilis* spores would be focused down to a 0.35 mm beam diameter (for 90% of the particles) or less. The aerodynamic lens consists of stainless steel tube

with multiple circular stainless steel lenses with small orifices that are separated by plastic spacers. Liu et al. invented the aerodynamic lens.[47, 49, 50] The theory of the aerodynamic lens is based on the fact that if particles are less than a critical value, they are moved closer to the axis by the lens. Multiple lenses increase this effect and results in a small beam diameter.

The spacers in this aerodynamic lens used in these experiments are the same as those designed by Seth Call, a previous member of the research group.[51] The lens calculator developed by Wang and McMurry was used to design new lens dimensions to focus down the range of sizes of *B. subtilis* spores instead of the original particle size range.[52, 53] Because the spores were unable to pass through the aerodynamic lens, they may have not fit the size requirement somehow. Our hypothesis is that the particles were too big at the beginning of the lens because they were not desolvated yet at the entrance to the vacuum chamber. Because the aerodynamic lens calculator assumes that the particle is the size entered at the beginning of the aerodynamic lens and stays constant throughout, the calculated lens dimensions were incorrect.

After switching to the beam tube, the bacteria were collected on most of the top sections of the Styrofoam collector even with no voltage applied to the deflection plates, which we interpreted that the beam of spores coming out of the beam tube was not well collimated. Because the bacteria were collected on the top sections of the Styrofoam collector, the acrylic collector was used again, but this time it was raised 3 mm higher with 5 size #6 washers. After the acrylic collector was raised, bacteria were collected only out of slot #3 when no voltage was applied to the deflection plates. This demonstrated that the beam was actually well collimated coming out of the beam tube, albeit in a different place than we thought it would be. Therefore,

there had to be an alternative explanation for the wide collection area previously observed for the Styrofoam collector. Polystyrene, which Styrofoam is made of, carries a negative charge when statically charged.[54] Because the spores had been positively charged from the electrospray, they were attracted to the entire surface of the Styrofoam. Previously, another group member had observed that spores bounce. During the charge detection experiments, the back end of the charge detector was swabbed and cultured and showed growth. This indicated that the spores had hit the back of the collection vessel and bounced onto the charge detector. In this case with the Styrofoam, we hypothesized that the spores had either bounced and been attracted back to the Styrofoam, or that they had hit the Styrofoam and then rolled around to another area of the Styrofoam.

From the results with both the Styrofoam collector and the original collector, we know that the *B. subtilis* spores survived positive electrospray conditions under vacuum, although the vacuum recovery rate (less than 0.1%) was lower than the atmospheric mobility recovery rate. The entrance into the vacuum system was much smaller than the entrance into the drift tube, so lower recovery rates were expected because fewer bacteria would make it into the system in the first place. Losses from electrospray to the end of a vacuum system are well documented and are consistent with our results.[55]

We did not observe much deflection of the spores with positive electrospray, even when the maximum voltage of -5000 V was applied to the right deflection plate. The furthest the spores was deflected was by one slot, into slot #4. The spores would have had less than approximately 63 charges (in units of e) and greater than 36 charges to be deflected this far.

These values were calculated using the previously derived equation for q in terms of y_{tot}. The y_{tot} was 0.44 and 0.25 inches respectively. The values used were V=5000V, L=0.011m, m=196 femtograms, v_x=50m/s, D=0.057m, and d=0.00635m. The value for v_x was taken from results of the earlier charge detection experiments. This result implies that either only the spores with a very low charge are surviving, or that the spores are not becoming as highly charged as expected from ESI. If the cause is that they are not becoming as highly charged, that may be because they are being sprayed in sterile (before the added spores) HPLC water. There may not be enough charge carriers in solution for the spores to become highly charged. In their virus studies, Siuzdak and Bothner reported that the amount of charge they observed on the viruses depended on the pH, but they did not provide information about what pH their electrospray solutions were.[12]

The other potential reason that we are not collecting any highly charged viable bacteria is that only those with a low charge state are surviving. We know from the atmospheric mobility experiments that the spores can survive at least some charge. In order to determine whether all the spores are in a low charge state or whether the spores have a variety of charge states and only the ones with little charge are surviving, more experiments are needed.

The bacterial spores also survived negative electrospray into vacuum conditions. They were also electrosprayed again in sterile HPLC water. Even with a maximum voltage of +5000V the spores were not deflected. This again implies that under these electrospray conditions either only the spores in a low charge state survive or none of the spores are acquiring high charges. The spores would have had between 0 and 36 negative charges to not be deflected at all. Because

the negatively electrosprayed spores were not deflected as much as the positively electrosprayed spores, we can hypothesize that either the negatively electrosprayed spores either had a lower number of charges than the positively electrosprayed spores, or they only survived with a lower number of charges than the positively electrosprayed spores.

4 CONCLUSION

4.1 Summary of Results

E. coli does not survive electrospray charging and desolvation. It does survive the aerosolization process of electrospray, but only when not fully desolvated, and thus not physically charged. *B. subtilis* spores do survive the electrospray charging and desolvation process even when de-agglomerated. They survive the process both at atmospheric conditions and under vacuum. The spores are either not highly charged when electrosprayed from a water solution, or only the spores in a low charge state survive.

4.2 Implications of Results

The discovery that bacterial spores survive electrospray charging could enable sorting of bacteria and mass spectrometry followed by culturing. It could also enable the use of electrospray as a source of charged bacteria that could be electrically accelerated for high velocity impact research. The fact that bacteria can survive electrospray charging opens up possibilities for different types of bacterial analyses to be coupled together. For example, electrospray can be used to introduce samples and separate bacteria by nondestructive mass spectrometry (MS) and ion mobility spectrometry (IMS) instruments followed by analysis using standard microbiological techniques. Additionally, the bacteria could possibly be manipulated and deposited into arrays separated by species. Such an approach would enable the use of a single small amount of sample in multiple steps of analysis.

This is also interesting in the context of exploring bacteria extremophile behavior. These experiments demonstrate another harsh condition that *B. subtilis* spores survive– being electrically charged in the gas phase. This characteristic can be added to the long list of extreme conditions that the spores can withstand.

4.3 Future Work

Further deflection experiments with different additions to the electrospray solvent such as salts or acid to attempt to get a higher charge state on the spores would be useful. If those are successful in producing more highly charged viable spores, then a larger charge distribution of the viable *B.subtilis* spores could be obtained.

If additions to the electrospray solvent do not result in more highly charged viable spores, then more experiments would be needed to determine if any of the bacteria are highly charged, but not viable. Possible experiments could include coupling a correctly calibrated charge detector with a deflector or using fluorescently tagged spores so that dead spores (if the fluorescent compounds stay in the cell upon death) could be detected as well.

With higher charged spores, electrostatic acceleration could be used for impact experiments. If spores are not able to be highly charged, then gas dynamic based acceleration may be preferable. Experiments testing the electrospray survivability of other species could be performed. If other species survive then their impact survivability could be tested as well.

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